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Screening of Microbial Isolates for the Fermentative Production of L-Asparaginase in Submerged Fermentation.

Kesavapillai Balakrishnan*, Aiswarya Nair and Rahul Kumar

Dept. of Biotechnology, Bannari Amman Institute of Technology – Sathyamangalam, Erode – 638401, TN, India

Research Article

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***Corresponding author**

Dept. of Biotechnology, Bannari Amman Institute of Technology – Sathyamangalam, Erode – 638401, TN, India
Mobile: +919943440346

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ABSTRACT

L-asparaginase is a well-recognized amino acid degrading enzyme exhibiting antineoplastic activity. It is desirable to opt for attempts leading to the discovery of new L-asparaginase producer that is serologically different from the previously reported ones, but exhibiting similar therapeutic effects. Twenty six microbial cultures including three microbial consortia were tested for their ability to produce L-asparaginase. Pure cultures of locally isolated microbes, a few commercially used microbes and three microbial consortia (gut consortia of earthworm and termites) were examined qualitatively and quantitatively for their ability to produce L-asparaginase. Seven of the 26 cultures including the sea water isolate tested, gave consistent positive results in qualitative assay and hence they were taken up for further quantitative studies. The cell free fermentation broth of the sea water isolate gave 0.710U /mL/ hour of L-asparaginase activity in starch casein broth. For a local isolate of S. cerevisiae (BIT -74) in yeast developmental broth, the same was found to be 0.884U /mL/hour. In M9 medium, when the yeast isolate was grown in the presence of asparagine, enzyme activity increases by a factor of 20%. The sea water isolates gave consistent enzyme activity in starch casein broth till 160 hrs and the pace of enzyme titer accumulation was high in asparagine incorporated M9 broth. It is clearly indicated through the findings in this study that many of the regular microbial cultures have the ability to produce L-asparaginase. A couple of isolates possess the ability to produce a significant amount of L-asparaginase enzyme. Presence of asparagine in M9 medium induced the enzyme yield.

INTRODUCTION

The enzyme L-asparaginase (L-asparagine amidohydrolase, E. C. 3.5.1.1) has attracted much attention as an amino acid degrading enzyme exhibiting antineoplastic activity [1,2,3]. Tumor cells are reported as taking in L-asparagine from blood circulation or body fluid as it cannot synthesize L -asparagines. The presence of L-asparaginase enzyme as chemotherapeutic agents may degrade the L-asparagine present in blood circulation and indirectly starve tumor cells and lead to cell death. Microbial enzymes are preferred over plant or animal sources due to their economic production, consistency, ease of process modification, optimization and purification. They are relatively more stable than corresponding enzymes derived from plants or animals [4]. A wide range of microorganisms such as bacteria, yeasts, and filamentous fungi have been regarded as ideal sources of L-asparaginase [5].

L-asparaginase has been used as a chemotherapeutic agent for over 30 years, mainly from the bacterial strains of *Escherichia coli* and *Erwinia chrysanthemi* [6,7,8]. Bacterial L-asparaginases sometimes cause allergic reactions and anaphylaxis [9] necessitating the search for other asparaginase sources and enzyme with less adverse effects. For the commercial production of enzyme, selection of superior strain and harvesting protocol ensuring sufficient quantity of enzyme are invariably crucial steps.

The administration of such an enzyme protein for a long duration, in general, produces the corresponding antibody in the tissues, resulting in anaphylactic shock or neutralization of drug effect. Therefore, the use of new serologically different L- asparaginase

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with a similar therapeutic effect is highly desirable [10]. Therefore, attempts leading to the discovery of new L-asparaginase producer that is serologically different from the previously reported ones, but one that has similar therapeutic effects is highly desirable and be encouraged.

MATERIALS AND METHOD

Materials

All the chemicals used were of analytical grade and procured from Rankem, New Delhi, Himedia, Mumbai and Merck, Mumbai, India. Eighteen microbial cultures used regularly for various studies in our laboratories were used in this investigation for the screening of L-asparaginase activity by growing them in respective maintenance media (nutrient broth for bacterial cultures, yeast development broth (YDB) for yeast cultures and Sabouraud dextrose broth (SDB) for fungal cultures. Besides these, five different microbial isolates which were locally isolated in our laboratory were also used in the screening of cultures for L-asparaginase activity. Two anaerobic consortia (collected and maintained from inside of Earthworm gut and termite gut) were also used along with another consortium maintained aerobically from earthworm gut for the screening of their ability to produce L-asparaginase.

Microorganisms

Eighteen microbial cultures (11 bacterial, 2 yeast and 5 fungal cultures) were used in the current investigation aimed at the ability of the cultures to produce L-asparaginase. Besides these standard cultures, five local isolates (3 yeast and 2 bacterial isolates) and three consortia (two anaerobic and one aerobic consortium) were also used in this investigations. The pure cultures were all maintained in their respective maintenance media (as stated above i.e. nutrient broth for bacteria, YDB for yeast cultures and SDB for fungal cultures) as agar slant cultures. Three yeast isolates (two (BIT-23 and BIT 74) were isolated from soil samples and one from sugarcane pressmud as per standard isolation procedures (serial dilution and plating)) were maintained in YD agar slants. Two local bacterial isolates (seawater isolate and oil cake isolate) were maintained on starch casein agar slants and nutrient agar slants respectively. Three consortia (two obtained from within earthworm gut and one from within termite gut using standard microbial isolation procedures) maintained separately in screw cap bottles were grown in molasses medium (specific gravity 1.020 with 1% (v/v) corn steep liquor). Aerobic consortium was in shaker (120 rpm) and anaerobic consortia were maintained as static cultures.

Qualitative plate assay for screening of L-asparaginase production

The isolates were screened for asparaginase activity using the method of Gulati *et al.* [11]. The fully grown cultures from slants (one loop full cultures in case of consortia) were plated in standard petriplates aseptically. The medium used was modified M_9 medium (pH 5.9) incorporated with a pH indicator (phenol red). The plates were incubated for 48 hours at 30°C. L-asparaginase activity was identified by formation of a pink zone around colonies.

Screening (quantitative) of microbial cultures for L -asparaginase activity

26 microbial cultures (including consortia) were initially grown in shake flasks (20 mL media held in 100 mL EM flasks) using respective culture media (Nutrient broth for bacteria, YDB for yeast cultures, SDB for fungal isolates, Starch Casein broth for sea water isolate and Corn medium for *Rhodosporidium*). Cultures were maintained at 120 rpm and at 30°C. The fermentation samples were harvested after 72 hours of growth and the cells were removed by centrifugation (5000 rpm, 10 min, 10°C). The cell free broths were tested for their ability to utilize asparagine as substrate in the asparaginase assay.

Determination of L-asparaginase activity (quantitative assay)

The method used was essentially that of Mashburn and Wriston [12]. In this assay, the rate of hydrolysis of L-asparagine was determined by measuring the ammonia released using Nessler's reaction. A mixture of 0.1 ml of enzyme extract, 0.2 ml of 0.05M Tris-HCl buffer (pH 8.6), and 1.7 ml of 0.01M L-asparagine was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1.5M trichloroacetic acid. After centrifugation at 10000 rpm, 0.5 ml of the supernatant was diluted to 7 ml with distilled water and treated with 1 ml of Nessler's reagent. The color reaction was allowed to develop for 10 min and the absorbance was read at 480 nm (against a reagent blank) using spectrophotometer (Shimadzu, Japan). The ammonia liberated was extrapolated from the standard curve derived with different concentrations of ammonium sulphate. One unit (IU) of L-asparaginase was defined as that amount of enzyme which liberates 1 µ mole of ammonia per hour under the assay conditions.

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Detection of peak L-asparaginase activity in cultures gave consistent positive results in plate assays

Seven cultures *Azotobacter sp., Azospirillum sp,* seawater isolate, *S. cerevisiae* BIT-74, *S. pombe,* pressmud isolate (yeast), *Pacelomyces sp.* that gave consistent plate assay color change were grown in 50 mL of the respective media (nutrient broth for bacterial cultures, YDB for yeasts and SDB for the fungal culture) held separately in 250 mL EM flasks. Two loops of the respective cultures from agar slants were used as the starter inoculum. They were grown at 30°C at 120 rpm for 166 hours. Periodic samples were derived and processed for quantitative L-asparaginase assay as mentioned above.

Comparative assessment of L-asparaginase production using selected microbial cultures

Four of the short listed cultures based on the performance of L-asparaginase production in their respective media over a period of time, were assessed for their enzyme titers till 72 h of culturing by growing them for the first time in the same medium. M9 medium (50 mL each in 250 mL EM flasks). Flasks were maintained at 120 rpm at 30°C for 72 hours. Samples were collected at 24 h intervals and processed for L-asparaginase activity as described earlier.

RESULTS

Qualitative plate assay for screening of L-asparaginase production

The release of ammonia from asparagine in M9 agar plates led to increase in local pH and hence the reddish appearance (due to the presence of phenol red indicator) of plates harboring positive cultures (Figure 1).

Figure 1: Plate assay indicating the release of ammonia from asparagine in M9 agar plates

Screening (quantitative) of microbial samples for L -asparaginase activity

In our investigation 16 out of 26 cultures tested gave considerable enzyme activity (more than 0.300 U/mL/hr.) in quantitative assay. Sea water isolates and local yeast isolate BIT74 gave the highest enzyme activities of 0.710 U/mL/hr and 0.884 U/mL/hr respectively (Table 1). None of the 5 fungal cultures tested, produced appreciable L-Asparaginase activity. Of the three consortiums tested, only termite gut consortium showed enzyme activity of 0.466 U/mL/hr.

Detection of peak L-asparaginase activity in cultures gave consistent positive results in plate assays

In order to find out the peak L-Asparaginase activity, seven select cultures from among the aforesaid 26 cultures were grown for up to 166 hrs. Sea water isolate showed consistent increase in enzyme titers till 160 hours in starch casein broth (Figure 2). The local yeast isolate BIT74, *S. pombe*, pressmud yeast isolates & *Paceliomyces* sp. showed enzyme activity during 72 hours in their respective media.

Comparative assessment of L-asparaginase production using selected microbial cultures

When the four of the select cultures grown in the same medium, M-9 broth containing asparagine as nitrogen source, the pace accumulation of enzyme titers was found to be high in all the cultures with sea water isolates exhibiting maximum enzyme activity at 48 hours (1.401 U/mL/hr.) (Table 2).

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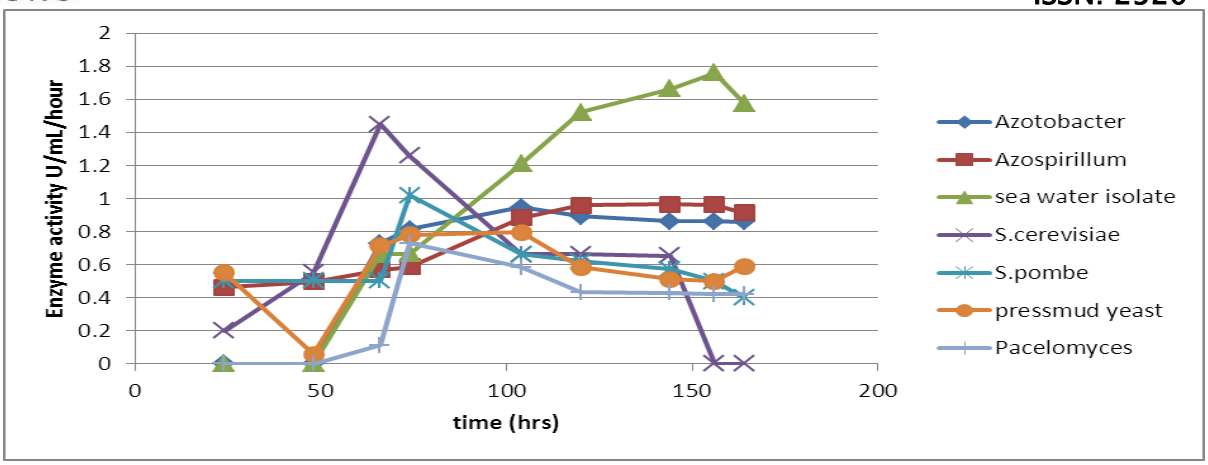

Figure 2: L-asparaginase production profile of selected microbial cultures in their respective maintenance media during submerged fermentation.

Table 1: L-asparaginase activities of various microbial isolates and cultures tested through quantification assay after 72 h of growth in their respective media

S. No	Microbial culture/ Isolates tested	Medium used for growing the culture	Enzyme activity (U/mL/h)
1	*Azotobacter* sp.	Nutrient broth	0.564
2	Azo*spirillum* sp.	Nutrient broth	0.512
3	*Pseudomonas* sp.	Nutrient broth	0.487
4	Phosphobacter (*Bacillus megatherium*)*	Nutrient broth	0.493
5	*Rhodosporidium* sp.	Corn medium	0.325
6	*Paceliomyces* sp.	Sabouraud Dextrose Broth	0.242
7	*Beauveria bassiana*	Sabouraud Dextrose Broth	0.002
8	*Rhizobium* sp.	Nutrient broth	0.509
9	*Thermomonospora sp.*	Streptomyces medium	0.011
10	*Saccharomyces pombe**	Yeast Development Broth	0.758
11	*S. cerevisiae*	Yeast Development Broth	0.590
12	Earthworm (aerobic) gut consortium	Molasses (1.020) with 1% corn	0.052
13	Earthworm (anaerobic) gut consortium	steep liquor (pH 6.8)	0.039
14	Yeast Local isolate BIT 23	Yeast Development Broth	0.613
15	Yeast Local strain BIT 74*	Yeast Development Broth	0.884
16	*Bacillus licheniformis*	Nutrient broth	0.522
17	*Bacillus subtilis*	Nutrient broth	0.344
18	Termites (gut consortium)	Same as used for 12 & 13	0.466
19	Pressmud yeast (isolate)	Yeast Development Broth	0.546
20	*Bacillus polymyxa*	Nutrient broth	0.524
21	*Myrothecium verrucaria*	Nutrient broth	0.223
22	Oilcake isolate	Nutrient broth	0.534
23	*Metarhizium* sp.	Sabouraud Dextrose Broth	0.226
24	*Aspergillus* sp.	Sabouraud Dextrose Broth	0.243
25	*Trichoderma* sp.	Sabouraud Dextrose Broth	0.142
26	Sea water isolate*	Starch Casein broth	0.710

Table 2: Production profile of L-asparaginase by select microbial cultures in M9 broth used for comparative assessment.

Samples	Enzyme activity (U/mL/h)			
	6 h	24 h	48 g	72 h
Azospirillum sp.	0.00	0.020	0.341	0.043
S. pombe	0.897	1.213	1.068	0.760
Sea water isolate	0.871	1.379	1.401	0.823
S. cerevisiae BIT-74	0.00	0.480	0.532	0.556

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DISCUSSION

Microorganisms are considered as untapped sources of metabolites and products with novel properties. They have a diverse range of catalytic enzymatic activity of various biochemical reactions. There is enormous scope for investigations aiming at exploring the possibilities of deriving new products of economic importance from potential organisms. The enzyme, L-asparaginase has been intensively investigated over the past two decades owing to its importance as antineoplastic agents. Like bacteria, actinomycetes and fungi are also good source for the production of L-asparaginase [13,14].

It is generally observed that L-asparaginase production is accompanied by an increase in pH of the culture filtrates. The plate assay was devised using this principle by incorporating the pH indicator phenol red in medium containing asparagine (sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing L-asparaginase. The plate assay is advantageous as the method is quick and L-asparaginase production can be visualized directly from the plates without performing time consuming assays [15,16,17].

Ambi rani *et al.* [18] reported the production of L-asparaginase in submerged fermentation wherein the maximum of 3.8 U/ml at 96 h of incubation period was attained. Further increases in incubation period didn't show any significant increase in enzyme production rather it was decreased. Thus optimum time of enzyme synthesis was 96 h after inoculation. This is in line with our findings in majority of the seven microbes tested through submerged culturing (see figure 02). The authors of this paper, when worked with *Aspergillus terreus* maximum enzyme activity was obtained when pomegranate skin powder was used as substrate in solid culturing at 120 h of cultivation [19] and okara hydrolysate fortified with L-asparagine gave 63.89 U/ml of activity at 120 h in submerged cultivation [20]. Sarquis *et al.* [13] reported that the highest L-asparaginase activity of *A. terreus* in liquid medium was found at 48 h while in solid medium the optimal period for enzyme production was 96 h. Only in sea water isolate, we found increased accumulation of enzyme activity till 160 hours of culturing (Figure 02).

Siddalingeshwara and Lingappa [21] indicated that the L-asparaginase production was maximum 6.05 IU at 72 hr fermentation period. While Khamna *et al.* [22] reported that the maximum L-asparaginase production was at pH 7.0 and temperature 30°C at 178 hr of fermentation period. This was more identical with our findings in starch casein broth for the sea water isolate which gave maximum enzyme activity at 160 hours.

The substrate incorporated M9 medium gave faster pace of accumulation of L-asparaginase activity in the cultures tested in our study. The enzyme production is reported as the result of complex chain reactions and is supported and induced by suitable substrates. *Penicillium sp.* preferred L-asparagines as substrate. This characteristic phenomenon of *Penicillium sp.* was corroborated with the Dunlope and Roon 1975 [23] who noted the increment in enzyme production due to the addition of L glutamine or glutamate in the fermentation medium. From this study, it is clearly indicated that many of the regular microbial cultures have the ability to produce L-asparaginase. A couple of isolates possess the ability to produce a significant amount of L-asparaginase enzyme. However, more detailed investigation is required to characterize this microbial enzyme, which may be effectively used in the large scale production for commercial and pharmaceutical purposes in the future.

Conflict of Interest

The authors have no conflict of interest to declare.

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REFERENCES

1. Bessoumy AA, Sarhan M, Mansour J. Production, Isolation, and Purification of L-Asparaginase from *Pseudomonas aeruginosa* 50071 Using Solid-state Fermentation. J Biochem Mol Biol. 2004; 37(4): 387-393.
2. Sahu MK, Sivakumar K, Poorani E, Thangaradjou T, Kannan L. Studies on L-asparaginase enzyme of *Actinomycetes* isolated from estuarine fishes. J Environ Biol. 2007; 28(2): 465-474.
3. Shah AJ, Karadi RV, Parekh PP. Isolation, optimization and production of L-asparaginase from *Coliform* Bacteria. Asian J Biotechnol. 2010; 2(3): 169-177.
4. Savitri NA, Azmi W. Microbial L-asparaginase: A Potent Antitumour Enzyme. Ind J Biotechnol. 2003; 2:184-194.

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5. Verma N, Kumar K, Kaur G, Anand S. L-asparaginase: a promising chemotherapeutic agent. Crit Rev Biotechnol. 2007; 27: 45-62.
6. Aghaiypour K, Farzami B, Mohammadi AA,Vand-Yoosefi J, Pasalar P, Safavieh S. Comparative study of L-asparaginase activity in different bacterial species. Arch Razi Inst. 1999; 50:51-58.
7. Aghaiypour K, Wlodawer A, Lubkowski J. Structuralbasis for the activity and the substrate specificity of *Erwiniachrysantemi* L-Asparaginase. Biochem. 2001; 40: 5655-5664.
8. Krasotkina J, Borisova AA, Gevaziev YV, Sokolov N. One-step purification and kinetic properties of the recombinant L-Asparaginase from *ErwiniaCarotovora.* Biotech Appl Biochem. 2004; 39: 215-221.
9. Sarquis M, Oliveira EMM, Santos AS, Costa GLD. Production of L-asparaginase by Filamentous Fungi, *MemInstOswaldo Cruz*. Rio de Janeiro. 2004; 99 (Suppl. 5): 489-492.
10. Tetsuya T, Ryujiro S, Kozo Y, Masatoshi N, Katsuka A, Ichiro CJ. L-Asparaginase from *Proteus vulgaris.* Appl Microbiol. 1971; 3: 387-392.
11. Gulati R, Saxena RK, Gupta R. A rapid plate assay for screening L-asparaginase producing microorganisms. Lett Appl Microbiolog. 1997; 24: 23-26.
12. Mashburn LT, Wriston JJC. Tumor inhibitory effect of L-Asparaginase from *Escheriachia coli*. Arch Biochem Biophys. 1964; 105: 450-452.
13. Sarquis MIM, Oliviera EMM, Santos AS, Da-Costa GL. Production of L-asparaginase by filamentous fungi. Mem Inst Oswaldo Cruz. 2004; 99: 489-492.
14. Prakasham RS, Rao CS, Rao RS, Lakshmi GS, Sarma PN. L-asparaginase production by isolated *Staphylococcus* sp. - 6A: Design of experiment considering interaction effect for processparameter optimization. J Appl Microbiol. 2007; 102: 1382-1391.
15. De-Jong P. L-Asparaginase production by *Streptomyces griseus.* Appl Microbiol. 1972; 23:1163-1164.
16. Wade HE, Robinson HK, Philips BW. Asparaginase and glutaminase activities of bacteria" J Gen Microbiol. 1971; 69: 299-312.
17. Arima K, Igarasi S, Nakahama K, Isono M. Production of extracellular L-asparaginases from microorganisms. Agr Biol Chem. 1972; 36: 356-361.
18. Ambirani S, Lalitha S, Praveesh BV. Isolation and screening of L-asparaginase producing fungi from soil samples. Int J Pharm Pharm Sci. 2012; 4: 279-282.
19. Nair A, Kumar R, Devi RA, Balakrishnan K. Screening of commonly available solid process residues as substrate for L-asparaginase production by *Aspergillus terreus* MTCC 1782. Res J Pharma Biol Chem Sci. 2013; 4 (2): 1731-1737.
20. Kumar R, Devi RA, Nair A, Balakrishnan K. Stimulatory Effect of Acid Hydrolysed Okara Fortified with L-Asparagine on Fungal L-Asparaginase Production by *Aspergillus terreus* MTCC 1782. Res J Pharma Biol Chem Sci. 2013; 4 (2): 1458-1468.
21. Siddalingeshwara KG, Lingappa K. Production and Characterization of L-Asparaginase- ATumour inhibitor. Int J Pharm Tech Res. 2011;3(Suppl.1): 314-319.
22. Khamna S, Akira Y, Saisamorn L. L-Asparaginase production by actinomycetes isolated from some Thai medicinal plant rhizosphere soil. Int J Integ Biol. 2009; 6(1): 22-26.
23. Dunlop PC, Roon RJ. L-asparaginase of *Sacharomyces cerevisiae*: an Extracellular Enzyme. J Bacteriol. 1975; 122: 1017-1024.